

**A NEW RECORD OF *Pyrostria* (VANGUERIEAE-RUBIACEAE)
FROM THE PHILIPPINES INFERRED FROM MOLECULAR
AND MORPHOLOGICAL DATA**

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ABSTRACT

Recent studies on *Canthium* revealed that the genus is polyphyletic. Subsequently, *Canthium sensu stricto* (s.s.) was re-delimited to species having paired supra-axillary spines, which led to the reinstatement of various genera in the Vanguerieae such as *Keetia*, *Psydrax* and *Pyrostria*. This raised questions on the generic affiliations of Philippine *Canthium* species as most of its members lacked spines. In an effort to revise the Philippine *Canthium*, the endemic spineless *Canthium subsessilifolium* was collected to ascertain its generic affiliation within the tribe using plastid (*trnL-F* region) and nuclear (ITS region) markers. The majority-rule consensus tree of combined data sets showed a well-supported clade of the whole Vanguerieae (PP=1.00). Interestingly, *C. subsessilifolium* was nested within the robustly supported *Pyrostria* clade (PP=1.00). Morphological similarities of *C. subsessilifolium* with *Pyrostria* further supported our molecular results. Therefore, a new combination is here provided: *Pyrostria subsessilifolia* (Merr.) Arriola & Alejandro. This study establishes for the first time the existence of *Pyrostria* in the Philippines.

KEYWORDS: *Canthium*, ITS, *Pyrostria*, Rubiaceae, *trnL-F*, Vanguerieae

INTRODUCTION

The Vanguerieae belongs to subfamily Ixoroideae of the Rubiaceae with 600 to 700 species of trees, shrubs, lianescent shrubs and geofructices that are distributed throughout tropical Africa, Madagascar, Asia and the Pacific (Razafimandimbison et al., 2009). Members of the tribe are characterized by a combination of axillary inflorescences, valvate corolla, a pendulous ovule, and a swollen styler knob (Lantz et al., 2002). Among the genera of the tribe, only *Canthium* Lam. is represented in the Philippines (Alejandro & Liede, 2003).

Canthium sensu lato (s.l.) is one of the most problematic groups of Vanguerieae since the presence of a 2-locular ovary is the only cardinal character used to delimit the genus (Verdcourt, 1987). As a result, *Canthium s.l.*

forms a heterogeneous group which contains elements that have more pronounced generic differences than many of the accepted genera in Vanguerieae (Bridson, 1985). Taxonomic efforts to evaluate the naturalness of *Canthium s.l.* utilizing either morphology or molecular data and a combination of both supported the reinstatement of various genera such as *Afrocanthium* (Bridson) Lantz and Bremer (Lantz & Bremer, 2004), *Bullockia* (Bridson) Razafim., Lantz & B. Bremer (Razafimandimbison et al., 2007), *Keetia* E.P. Phillips, *Psydrax* Gaertn., and *Pyrostria* Commers. ex Juss. (Bridson, 1985; 1986; 1987). Consequently, *Canthium sensu stricto (s.s.)* was recircumscribed to include species having supra-axillary spines (Lantz & Bremer, 2004). In the course of taxonomic revision of *Canthium s.s.* (Bridson, 1992), only species from Africa and Madagascar received attention. Conversely, there are numerous Asian species of *Canthium* including Philippine representatives left unresolved. In the Philippines, there are 20 currently recognized species of *Canthium* whose delimitation is still based on the work of Merrill (1928). As most of the endemic Philippine *Canthium* are without spines, their generic affinities should be re-examined.

Modern taxonomy utilizes molecular data like the internal transcribed spacer (ITS) and the *trnL-F* from the nuclear and chloroplast genomes, respectively, to better understand the phylogenetic relationships of plant species (Golovnina et al., 2007). The ITS region (ITS1 and ITS2) is located in between the 18S and 26S nrDNA and is flanked by a highly conserved 5.8S gene (Baldwin, 1992). The *trnL-F*, on the other hand, is a part of the group I intron and located within the large single copy region of the cpDNA. This non-coding region is associated with the highly conserved transfer RNA genes for leucine (UAA) and phenylalanine (GAA) that are found in tandem and is separated by spacers of several hundred base pairs (bp) (Borsch et al., 2003). Both markers are very useful in reconstructing phylogeny at the lower taxonomic ranks (Douzery et al., 1999).

In this study, the ITS and *trnL-F* regions of the Philippine endemic *C. subsessilifolium* (Merr.) Merr. were sequenced to determine its affiliation in the context of the present-day generic concepts in the Vanguerieae. In addition, a species description, botanical illustration and conservation status assessment of the species are here provided for the first time. This study is a pioneering effort towards the revision of the whole Philippine Vanguerieae.

MATERIALS AND METHODS

Taxon Sampling. This study was based on field observation and collection of *C. subsessilifolium* from Balangiga, Eastern Samar (11°07'N, 125°23'E). Furthermore, herbarium sheets from L and PNH as well as digital images from HUH, K and US were observed. Flowering branches were collected for herbarium vouchers while pickled reproductive parts were preserved in 70%

ethanol. Leaf samples were placed in zip-lock bags containing silica gel for molecular analysis (Chase & Hills, 1991).

Molecular Methods. Total genomic DNA was extracted from silica gel-dried leaf tissues using the DNeasy Plant Minikit (Qiagen, Germany). The ITS and *trnL-F* sequences for species of Vanguerieae were obtained from Genbank, thus, these primers were selected for this study. The entire ITS region (including the 5.8S gene) was amplified and sequenced using the primers P17F/26-82R and P16F/P25R, respectively (Popp & Oxelman, 2001). Meanwhile, primers c/f were used for both the amplification and sequencing of the *trnL-F* region (Taberlet et al., 1991). DNA amplification was carried out following the work of Alejandro et al. (2005, 2011). Amplified DNA was purified using the QIAquick Purification Kit (Qiagen, Germany). Purified DNA was sent to MACROGEN Inc. Seoul, South Korea for sequencing.

Sequence Alignment and Phylogenetic Analysis. DNA sequences for the ITS and *trnL-F* were assembled and edited using the CodonCode Aligner v3.0.1. Edited sequences of *C. subsessilifolium* were manually aligned together with the previously published sequences of Vanguerieae from the work of Lantz and Bremer (2004) and Razafimandimbison et al. (2009) using Se-Al v.1.0a1 (Rambaut, 1996). *Ixora* species and *Mussaenda erythrophylla* were used as outgroups following the work of Lantz and Bremer (2004). Table 1 shows the new accession numbers of *C. subsessilifolium* (ITS: HG426456, *trnL-F*: HG426459) including the list of accessions for other taxa included in the analyses.

Bayesian inference (BI) was used to estimate phylogenetic position of *C. subsessilifolium*. The analysis was carried out using the MrBayes v.3.1.2p software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Altekar et al., 2004). Model selection for the best-performing evolutionary models were determined under three model selection criteria: 1) Akaike Information Criterion (AIC) (Akaike, 1974), 2) AICc (second order criterion of AIC, necessary for smaller samples) and 3) the Bayesian Information Criterion (BIC) (Schwartz, 1978). The selected models were HKY and GTR + G for the ITS and *trnL-F*, respectively. In analyzing single marker, the best performing model was selected and one million generation was considered with a sample frequency of 1000 and four parallel chains. For combined analyses, model selection as well as the settings is similar with that of the single-marker analysis, however there were a total of three million running generations. Clades with posterior probability (PP) exceeding 0.95 were regarded as strongly supported.

To compare the topologies inferred from Bayesian inference, parsimony analysis was conducted using PAUP version 4.0b (Swofford, 2000). Most parsimonious trees were determined using heuristic search, tree-bisection

reconnection (TBR) branch swapping using 10,000 random addition sequences, with MULTREES option on. Consistency index (Kluge & Farris, 1969) and retention index (Farris, 1989) were calculated to determine if the data is far from being homoplasious. Bootstrapping was determined using 10,000 replicates, MULTREES option off, TBR branch swapping, and five random addition sequences. Clades receiving a bootstrap (BS) value greater than 90% were considered strongly supported.

Table 1. Nucleotide sequence database accession numbers of taxa used in phylogenetic analysis

Taxon	ITS	<i>trnL-F</i>
<i>Afrocanthium burtii</i> (Bullock) Lantz	AJ617749	AJ620120
<i>Afrocanthium gilfillanii</i> (N.E. Br.) Lantz	AJ617751	AJ620123
<i>Afrocanthium keniense</i> (Bullock) Lantz	AJ617753	AJ620126
<i>Afrocanthium lactescens</i> (Hiern) Lantz	AJ617754	AJ620127
<i>Afrocanthium mundianum</i> (Cham. & Schltdl.) Lantz	AJ315107	AJ620128
<i>Afrocanthium parasiebenlistii</i> (Bridson) Lantz	AJ617756	AJ620130
<i>Afrocanthium pseudoverticillatum</i> (S. Moore) Lantz	AJ617758	AJ620132
<i>Afrocanthium siebenlistii</i> (K. Krause) Lantz	AJ617759	AJ620133
<i>Canthium ciliatum</i> (D. Dietr.) Kuntze	AJ617750	AJ620121
<i>Canthium coromandelicum</i> (Brum. f.) Alston	AJ315081	AJ620122
<i>Canthium confertum</i> Korth.	EU584288	-
<i>Canthium glaucum</i> Hiern ssp. <i>glaucum</i>	AJ617752	AJ620124
<i>Canthium inerme</i> (L.f.) Kuntze	AJ315120	AJ620125
<i>Canthium mrimaense</i> (Verdc.) Lantz	AJ617775	AJ620174
<i>Canthium subsessilifolium</i> (Merr.) Merr.	HG426456	HG426459
<i>Cyclophyllum balansae</i> (Baill.) Guillaumin	EU584357	EF205630
<i>Cyclophyllum barbatum</i> (G. Forst.) N. Halle & J. Florence	EU584356	AJ630135
<i>Cyclophyllum merrillianum</i> Guillaumin	-	FJ150540
<i>Fadogia ancylantha</i> Schweinf.	AJ315103	AJ620136
<i>Fadogia arenicola</i> K. Schum. & K. Krause	AJ874981	AJ874943
<i>Fadogia tetraquetra</i> K. Schum. & K. Krause	AJ315099	AJ620139
<i>Fadogia triphylla</i> Baker	AJ874982	AJ874944
<i>Keetia gueinzii</i> (Sond.) Bridson	AJ315117	AJ620143
<i>Keetia lukei</i> Bridson	AJ617761	AJ620144
<i>Keetia venosa</i> (Oliv.) Bridson	AJ617762	AJ620145
<i>Keetia zanzibarica</i> (Klotzsch) Bridson ssp. <i>Zanzibarica</i>	AJ315105	AJ620138
<i>Psydrax nitida</i> (Craib) Wong	AJ315108	AJ620160
<i>Psydrax obovata</i> (Klotzsch ex Eckl. & Zeyh.)	AJ315109	AJ620161

Bridson ssp. <i>obovata</i>		
<i>Pyrostria ampijoroense</i> (Arènes) Razafim., Lantz & B. Bremer	AJ617766	AJ719194
<i>Pyrostria commersonii</i> J.F. Gmel.	FN386385	-
<i>Pyrostria hystrix</i> (Bremek.) Bridson	AJ315114	AJ620168
<i>Pyrostria major</i> (A. Rich. ex DC.) Cavaco	EU584304	FN386344
<i>Pyrostria orbicularis</i> A. Rich. ex DC.	EU584285	FN386347
<i>Pyrostria phyllantoidea</i> (Baillon) Bridson	AJ315115	AJ620169
<i>Pyrostria revoluta</i> (Balf. f.) Razafim., Lantz & B. Bremer	AJ617776	AJ620176
<i>Pyrostria sarodranensis</i> Cavaco	EU584280	FN386366
<i>Pyrostria serpentina</i> Lantz, Klack. & Razafim.	EU584283	FN386350
<i>Pyrostria viburnoides</i> (Baker) Verdc.	EU584286	FN386355
<i>Vangueria infausta</i> Burchell	AJ617777	AJ620180
<i>Vangueria proschii</i> Briq.	AJ875009	AJ874975
<i>Vangueria parvifolia</i> Sond.	AJ315092	AJ620181
<i>Ixora coccinea</i> L.	AJ224826	AJ620117
<i>Ixora finlaysoniana</i> Wall. ex G. Don	-	AY555085
<i>Ixora trichocalyx</i> Hochr.	-	FJ150605
<i>Mussaenda erythrophylla</i> Schumach. & Thonn.	AJ224823	AJ620116

RESULTS AND DISCUSSION

Sequence Characteristics. The aligned ITS and *trnL-F* data matrices contained a total of 851 and 1,002 included characters, respectively. The ITS region had 273 parsimony-informative sites while 57 parsimony-informative characters were recorded for the *trnL-F* dataset. The combined ITS-*trnL-F* data matrix resulted to a total of 330 informative characters from the 1,853 included positions (Table 2). The consistency and retention index of both the separate and combined datasets ranged from 0.60 to 0.93, which confirmed that data are far from being homoplasious and thus reliable in reconstructing phylogenies.

Table 2. Matrix characteristics of separate and combined datasets

	ITS	<i>trnL-F</i>	Combined Data
Number of Taxa	42	43	45
Number of Included characters	851	1,002	1,853
Number of Informative Characters	273	57	330
Consistency Index	0.60	0.93	0.65
Retention Index	0.75	0.93	0.77

Phylogenetic Analysis. The majority rule consensus tree (Figure 1) of the combined ITS-*trnL-F* showed a highly supported monophyletic Vanguerieae (PP=1.00, BS=100%). The phylogenetic positions of the included genera (*Afrocanthium* (Bridson) Lantz & B. Bremer, *Canthium* s.s., *Fadogia* Schweinf., *Keetia* E. Phillips, *Psydrax* Gaertn., *Pyrostria* Comm. ex A. Juss. and *Vangueria* Juss.) were also strongly supported corroborating the results of Lantz and Bremer (2004). The Southeast Asian *Canthium confertum* is closely related to the New Caledonian *Cyclophyllum* (PP=1.00, BS=51%) and forms a sister clade with *Pyrostria* s.s. (PP=1.00, BS=82%) conforming to the results of Razafimandimbison et al. (2009). *Canthium subsessilifolium* did not group with *Canthium* s.s. but instead is closely related to *Pyrostria* s.s. (PP=0.58).

The phylogenetic position of *C. subsessilifolium* with another genus other than *Canthium* is expected due to its lack of spines thus not fitting to the current circumscription of the genus. Although our molecular data showed low support, Bridson (1987) has previously hinted the relatedness of *C. subsessilifolium* with *Pyrostria*. Our molecular results served to confirm her hypothesis on the close affinity of *C. subsessilifolium* with *Pyrostria*. In her taxonomic work she placed *C. subsessilifolium* under *Pyrostria* Group 1B, a group of bracteate Southeast Asian *Canthium*. However, she did not make any nomenclatural changes due to geographical reasons. Furthermore, Bridson (1987) was clear that the two species, *C. subsessilifolium* and *C. confertum*, both occurring in the Malesian region, are distinct from each other and placed the latter under *Pyrostria* s.l. Group IV composed mainly of ebracteate Southeast Asian *Canthium*. Meanwhile, apart from the presence of a persistent pair of connate bracts, our morphological investigation clearly showed that *C. subsessilifolium* is indeed related to *Pyrostria* than with any other genera of the Vanguerieae due its dioecious sexuality and fleshy corollas with large numbers of moniliform hairs (Lantz & Bremer, 2004). Recently, Razafimandimbison et al. (2009) established new generic limits within *Pyrostria* based on dioecious sexuality and the presence of persistent pair of bracts. This recircumscription led to the lumping of Bridson's *Pyrostria* Group 1A (*Leroya* Cavaco and *Neoleroya* Cavaco) with *Pyrostria* s.s. Unfortunately, species under *Pyrostria* Group 1B were not included due to lack of material. However, Razafimandimbison et al. (2009) mentioned that these Southeast Asian species would probably belong to the newly delimited *Pyrostria* s.s. based on the distinct features presented above. As a matter of fact, *Canthium brunnescens* Craib and *C. cochinchinensis* Pierre ex Pitard have been nomenclaturally transferred to *Pyrostria* by Utteridge and Davis (2009).

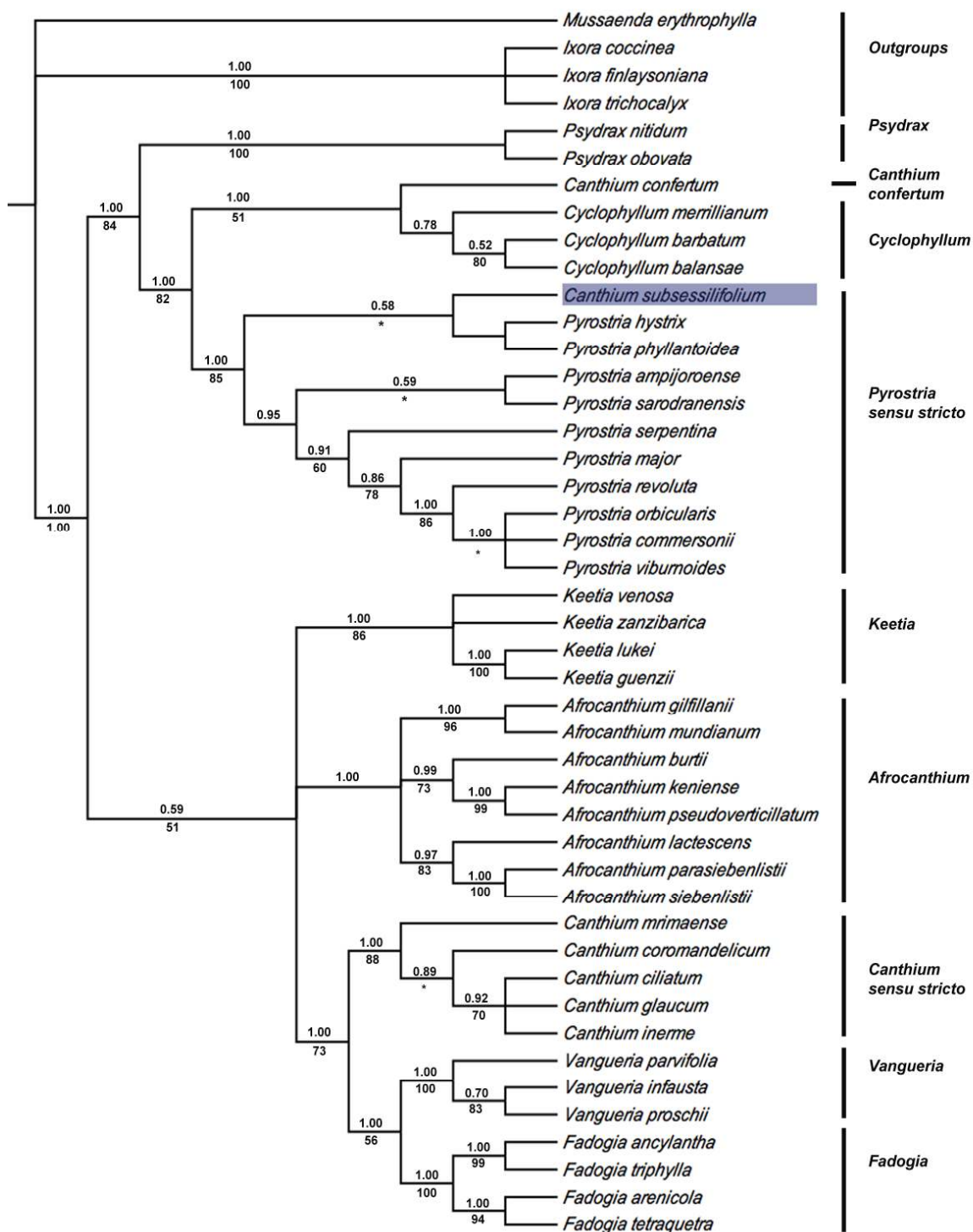


Figure 1. Majority-rule consensus from the Bayesian analysis of the combined ITS-*trnL-F* data set of 44 included taxa. These results were congruent with parsimony analysis, except with nodes marked by an *asterisk*. Numbers above branches indicate Bayesian posterior probabilities and those below are parsimony bootstrap support values. *Canthium subsessilifolium* is highlighted in grey.

TAXONOMIC TREATMENT

Since our molecular result is strongly supported by morphological data we propose a new combination:

***Pyrostria subsessilifolia* (Merr.) Arriola and Alejandro, comb. nov.** – Figure 2. Basionym: *Plectronia subsessilifolia* Merr. Philipp. J. Sci. 1917. 12: 168. Homotypic synonym: *Canthium subsessilifolium* (Merr.) Merr. Philipp. J. Sci. 1928. 35: 9. TYPE: Philippines, Burgos, Ilocos Norte, 02 Mar 1917, Ramos and Edano 27205 (Holotype, PNH, presumably destroyed; Lectotype, designated here, K! <1 sheet> ; isoelectotype, US! <1 sheet>, HUH!<1 sheet>).

Shrubs to small trees, up to 3 m high; branches terete and glabrous. Leaves elliptic to obovate, 6.8–12.4 × 3.2–5.4 cm, coriaceous to subcoriaceous, glabrous on both sides; apex obtuse to broadly acuminate; base acute; lateral nerves 3 to 5 on each side of the midrib; petiole subsessile, 1.0–2.0 mm, glabrous. Stipule ovate to triangular 2.0–4.0 × 1.5 mm, glabrous on both sides.

Inflorescence axillary, 3 to 5 flowers arranged in pedunculate umbels. Peduncle subsessile to 1.0 mm long, glabrous. Bracts present 1.0–1.5 × 1.0 mm, glabrous on both sides. Flower pedicels 3.0–5.0 mm long, glabrous to sparsely pubescent. Calyx tube yellow-green, 1.0–2.0 × 2.0 mm, glabrous inside, shortly dentate. Calyx lobes five, 0.2–0.3 × 0.3 mm, apex triangular, glabrous on both sides. Corolla tube infundibular 2.0–4.0 × 2.0–4.0 mm glabrous outside, puberulous inside. Corolla lobes fleshy, 1.0–1.2 × 0.9–1.0 cm, triangular, glabrous outside, puberulous inside. Female flowers: style 6.0–9.0 mm long, stigmatic knob 2.0–3.0 mm. Ovary 2-locular. Male flowers not seen. Fruits axillary, one to two per peduncle, yellow, obovoid, about 7.0 × 5.5 mm long.

Distribution. Distributed in Ilocos, Bataan and Samar. The species thrives in lowland forest around 200–350 masl.

Phenology. Flowering May - June: Fruiting June - October

Additional Specimen studied. **PHILIPPINES.** **Luzon:** Bataan Prov., Mt Mariveles, 14°26'N, 120°29'E, Jan. 2011, *Arriola, Poblete and Banag TA021* (USTH); Municipality of Morong, Barangay Kanawan, 14°43'N, 120°22'E, Feb. 2011, *Arriola T025* (USTH); Ilocos Norte Prov., Mangoratao (no specific coordinates provided), Aug. 1992, *FJM Gaerlan et al. 9867* (L, PNH). **Visayas:** Eastern Samar Prov., Balangiga, 11°07'N, 125°23'E, May 2012, *Lemana, Sonata, Poblete Uy 12BC008* (USTH); Samar Prov., Municipality of Paranas, Camp Uno, 11°51'N, 125°06'E, May 1996, *Reynoso and Majaducon 24108* (L, PNH).

Conservation Status. The Philippine Plant Inventory (PPI) project of the Philippine National Museum and B.R.I.T for the Flora of the Philippines piloted an exhaustive botanical exploration during the early 90's. In the course of revising Philippine Vanguerieae, most of the duplicate sheets from the PPI collections deposited in L were examined and indet collections were identified.

In effect, *P. subsessilifolia* was accounted only in Ilocos Norte and Central Samar. Furthermore, current Rubiaceae collections conducted by the Thomasian Angiosperm Phylogeny and Barcoding Group (TAPBG) on key forested regions of the Philippines neither observed nor collected *P. subsessilifolia* except in the aforementioned provinces with less than 2 to 3 mature individuals growing in the wild. These accounts on *P. subsessilifolia* clearly shows its restricted distribution, thus based on the criteria of the IUCN (2001), we assessed *P. subsessilifolia* as endangered (EN B1).

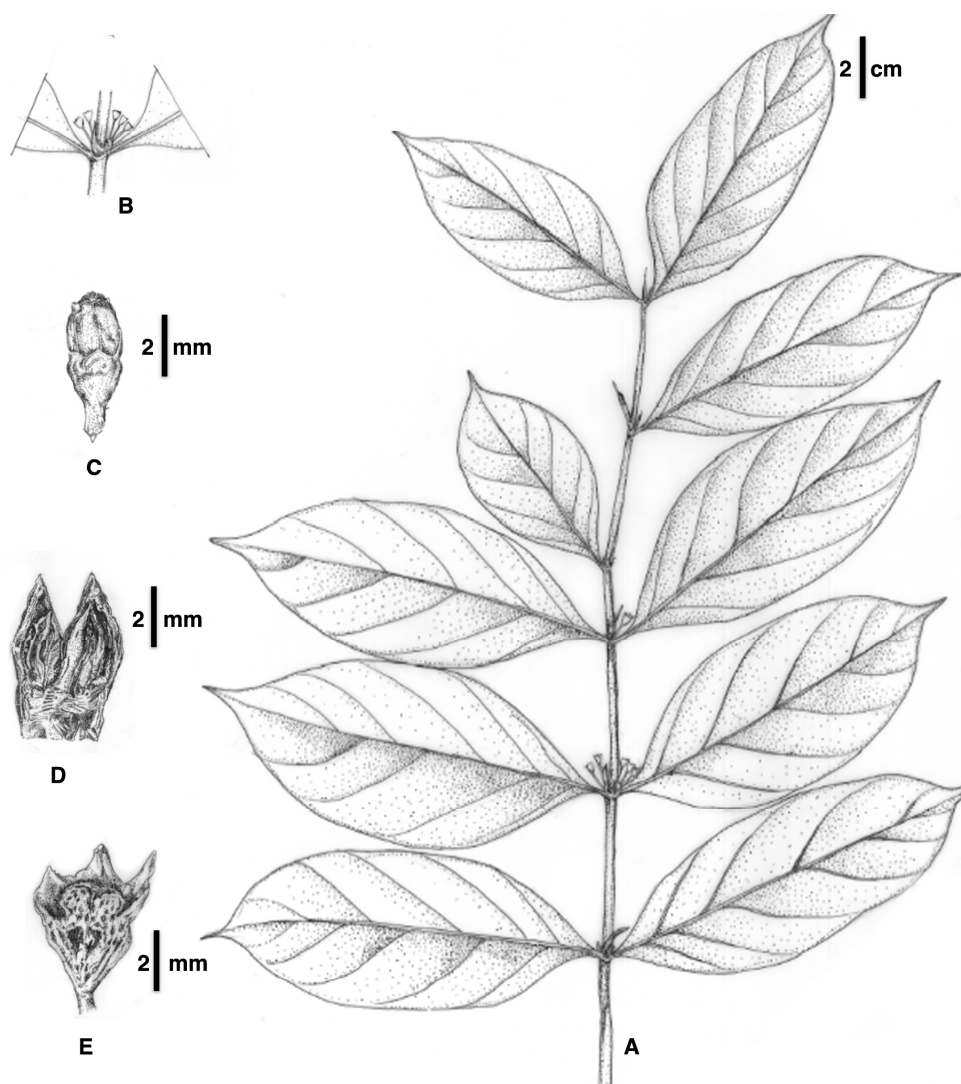


Figure 2. *Pyrostria subsessilifolia* (Merr.) Arriola & Alejandro, *comb. nov.* A. flowering branch; B. inflorescence showing the stipule; C. flower bud; D. opened corolla showing moniliform hairs; E. ovary.

CONCLUSIONS

Bayesian inference analysis utilizing the combined ITS (rDNA) and *trnL-F* (cpDNA) and morphological data set supports for the first time the phylogenetic position of *Canthium subsessilifolium* within the *Pyrostria* clade. Thus a new combination *P. subsessilifolia* (Merr.) Arriola & Alejandro *comb. nov.* is proposed in this study, making this the first account of the genus *Pyrostria* in the Philippine flora.

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